# A STUDY OF SOME OF THE FACTORS INVOLVED IN THE BLOOD CLOTTING MECHANISM

BY

KENNETH CARL ROBBINS

B. S., University of Illinois, 1939

M. S., University of Illinois, 1940

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
PHYSIOLOGICAL CHEMISTRY IN THE
GRADUATE SCHOOL OF THE
UNIVERSITY OF
ILLINOIS, 1944

CHICAGO, ILLINOIS
1944

*The author wishes to express his appreciation to*

Dr. William H. Welker

*for his advice and assistance in this investigation.*

# A STUDY OF SOME OF THE FACTORS INVOLVED IN THE BLOOD CLOTTING MECHANISM

BY

KENNETH CARL ROBBINS

B. S., University of Illinois, 1939

M. S., University of Illinois, 1940

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
PHYSIOLOGICAL CHEMISTRY IN THE
GRADUATE SCHOOL OF THE
UNIVERSITY OF
ILLINOIS, 1944

CHICAGO, ILLINOIS
1944

# A STUDY OF SOME OF THE FACTORS INVOLVED IN THE BLOOD CLOTTING MECHANISM

The blood clotting mechanism can be divided into two phases: precoagulation and coagulation. The precoagulation phase involves the interaction of prothrombase, calcium ions and thromboplastin with the formation of thrombase, the active clotting agent. The coagulation phase involves the action of thrombase in converting fibrinogen to fibrin. The purpose of this investigation is to study the relationship between the various factors involved in blood clotting, utilizing chemical, physical and immunologic methods. These experiments have been performed with the purpose of trying to more fully understand the mechanism of conversion in both phase I and II.

The precipitation of prothrombase and associated plasma proteins by a weak acid gives the most active preparation of thrombase. A rapid, reliable, modified Mellanby method for the preparation of prothrombase and thrombase is described. A specially prepared phosphate buffer containing calcium ions is used to extract the prothrombase from the associated plasma proteins. The prothrombase is converted into thrombase by allowing the wet prothrombase precipitate to stand at room temperature for 24 hours (pH 5.0-5.1). To prepare prothrombase this precipitate is immediately dried with acetone. The thrombase activity ranges from 101-402 units per mg. of N. One unit of activity is the amount of thrombase required to clot 1 cc. of fresh oxalated beef plasma in 30 seconds at 37°C. This method is applicable to oxalated and citrated beef and oxalated sheep plasmas, but is not applicable to oxalated or citrated human or oxalated hog plasmas.

Thrombase solutions were fractionated with a saturated ammonium sulfate solution, the activity was found in the 46-64% fractions; the most active preparation contained 1000 units per mg. of N. Solutions of thrombase can be stored at 4°C. for months when preserved with merthiolate 1-10,000 without any appreciable loss in activity.

Mellanby and Milstone have postulated a "spontaneous" activation of prothrombase to thrombase. This "spontaneous" ac-

tivation has been shown to be due to calcium ions. It was found that calcium is necessary for the in vitro activation of prothrombase; oxalate inhibits this activation. The purification of prothrombase presents difficulties due to the activation of precipitates which sometimes contain calcium or thromboplastin or both. Solutions of prothrombase can be stored at 4°C. when preserved with merthiolate 1-10,000. Calcium ions will activate prothrombase after long periods of storage (14 weeks).

Prothrombase solutions were fractionated with a neutral saturated ammonium sulfate solution (pH 7.0). This fractionation produced thrombase. To eliminate the possibility of calcium ions in this activation, potassium oxalate was added to the prothrombase solution, before fractionation, in a final concentration of 0.2%. The oxalate did not inhibit the formation of thrombase by fractionation of prothrombase with neutral ammonium sulfate. The activities found in both experiments were comparable and approximately equal. Calcium is therefore not the agent responsible for this activation.

This fractionation with neutral ammonium sulfate solutions may have split a prothrombase complex, liberating the active clotting agent (thrombase). It is possible that this salt effect is similar either to the activation produced by calcium ions and thromboplastin, or the activation produced by proteolytic enzymes, i.e., trypsin. The inactive prothrombase molecule is activated by any agent that has the power of splitting fibrinogen from the complex and leaving the rest of the molecule intact.

In the investigations previous to the one described here, no chemical relationships were established between prothrombase and thrombase. The differentiation between these two compounds was based solely on activity or solubility of the protein combinations. The immunologic reactions of prothrombase and thrombase, when considered with the results of our chemical and physical studies, lead to the establishment of a definite relationship between these two substances.

Specific precipitin antisera were prepared in rabbits against beef prothrombase and thrombase by intramuscular injections of these antigens adsorbed on aluminum hydroxide, according to the method described by Hektoen and Welker. Prothrombase

produces precipitins which react with prothrombase, thrombase, fibrinogen and euglobulin. Thrombase produces precipitins which react with prothrombase, thrombase and euglobulin. Prothrombase contains at least two plasma proteins, fibrinogen and euglobulin; thrombase contains euglobulin but not fibrinogen.

In vitro adsorption experiments will not differentiate between euglobulin and thrombase since all the precipitins are adsorbed from a thrombase precipitin serum by euglobulin. In order to determine the species specificity of thrombase, several thrombase precipitin sera were titrated against beef plasma and serum, human, dog, hog and sheep sera. The thrombase antisera reacted only with the homologous blood proteins (beef plasma, serum and euglobulin). This experiment indicates that the antigen or antigens in thrombase are species specific. This may mean that euglobulin is the carrier for the enzyme and therefore would be specific for each species of blood used although the coagulation activity is not specific.

One can conclude from these experiments that prothrombase is a fibrinogen-euglobulin-thrombase complex and is converted into an active clotting agent (thrombase) by splitting off the fibrinogen from the complex. Similar results were obtained in the fractionation of prothrombase solutions with a neutral saturated ammonium sulfate solution. At 25% saturation with ammonium sulfate, the fibrinogen is split off the prothrombase complex producing thrombase. The 0-25% fraction came down as a typical fibrin clot.

Thrombase behaves like an albumin in its physical and chemical properties but immunologically it shows the reactions of euglobulin and perhaps some other protein.

In the course of our investigations on fibrin, it was observed that there were some differences in the properties of fibrin obtained from defibrinated blood or recalcified plasma (Ca-fibrin) and that obtained when oxalated or citrated plasma is clotted with thrombase (T-fibrin). Ca-fibrin is insoluble but swells to a jelly in 0.03% hydrochloric acid and is insoluble in 0.5% sodium carbonate. T-fibrin is soluble in these reagents. The precipitate which forms during the storage of oxalated plasma has the solubility of T-fibrin. The clotting of oxalated or citrated

plasma by trypsin, Fer de Lance venom, clotting globulin and ninhydrin produce T-fibrin; these fibrins are soluble in 0.03% hydrochloric acid.

The possibility existed that the clotting mechanism proceeded in two directions, either to produce Ca-fibrin or T-fibrin, but since Ca-fibrin is the end product of the in vivo mechanism, perhaps the T-fibrin is only an intermediate in the conversion of fibrinogen to Ca-fibrin. It was found that, in vitro, calcium ions will convert T-fibrin into Ca-fibrin. This effect is not a specific one, strontium ions will also produce this conversion but barium or magnesium ions will not. An enzyme is probably not involved in this change. It is possible that the Ca-fibrin may be a calcium compound.

A study of the conversion of fibrinogen to fibrin using purified reagents has been the aim of all investigators. In mixtures containing purified fibrinogen, thrombase and calcium, it was found that a serum factor besides the calcium ions is necessary for the in vitro formation of Ca-fibrin. This factor is not present in the purified serum protein fractions and may have been destroyed or removed in their isolation. It was also found that Fer de Lance venom could not replace thrombase in the purified clotting system and is therefore not a thrombase-like compound. Clotting globulin can replace thrombase in the purified clotting system and can be considered as a thrombase-like compound. The following equations illustrate the in vitro mechanism in the conversion of fibrinogen to fibrin:

Fibrinogen + Thrombase ⟶ T-Fibrin

T-Fibrin + Ionic Calcium + Serum factor ⟶ Ca-Fibrin

In our immunologic studies on fibrinogen and fibrin, it was found that heat coagulated fibrinogen, Ca-fibrin, T-fibrin and heat coagulated Ca-fibrin produced precipitins for fibrinogen. Heat coagulation of fibrinogen and the fibrins changes their solubilities in 0.03% hydrochloric acid and 0.5% sodium carbonate. From these results, it would appear that the antigenic nucleus remains unaffected by clotting or by heat denaturation of fibrinogen, regardless of its change in solubility. The antigenic nucleus may be a very stable one. These results also indicate

that the antigenic groups in the molecule were not changed in the conversion of fibrinogen to fibrin. The fibrinogen and fibrin molecules do not contain either albumin or pseudoglobulin.

## SUMMARY

1. A rapid, reliable modified Mellanby method for the preparation of thrombase is described. This method is applicable to oxalated and citrated beef and oxalated sheep plasmas but is not applicable to oxalated or citrated human or oxalated hog plasmas.

2. When thrombase is fractionated with ammonium sulfate, the activity is found in the 46-64% fraction; the most active preparation contains 1000 units per mg. of N.

3. Calcium is necessary for the in vitro activation of prothrombase to thrombase; oxalate inhibits this activation. The "spontaneous" activation of prothrombase has been shown to be due to calcium ions.

4. Fractionation of prothrombase with neutral ammonium sulfate produces thrombase. Calcium ions are not necessary in this reaction.

5. Prothrombase produces precipitins which react with prothrombase, thrombase, fibrinogen and euglobulin. Thrombase produces precipitins which react with prothrombase, thrombase and euglobulin. Prothrombase contains at least two plasma proteins, fibrinogen and euglobulin; thrombase contains euglobulin, but not fibrinogen.

6. In vitro adsorption experiments will not differentiate between euglobulin and thrombase.

7. The antigen or antigens in thrombase are species specific.

8. Prothrombase is a fibrinogen-euglobulin-thrombase complex and is converted into an active coagulating agent (thrombase) by splitting off the fibrinogen.

9. Two different fibrins have been demonstrated based on their solubility in dilute acids and alkalies. Ca-fibrin is insoluble but swells to a jelly in 0.03% hydrochloric acid and is insoluble

in 0.5% sodium carbonate. T-fibrin is soluble in these reagents.

10. The clotting of oxalated or citrated plasma by thrombase, trypsin, Fer de Lance venom, clotting globulin and ninhydrin produce T-fibrin; these fibrins are soluble in 0.03% hydrochloric acid.

11. Calcium ions are necessary for the in vitro formation of Ca-fibrin. Calcium ions convert T-fibrin into Ca-fibrin. This effect is not a specific one, since strontium ions will also produce this conversion but barium or magnesium ions will not. An enzyme is probably not involved in this change.

12. A serum factor besides the calcium ions is also necessary for the in vitro formation of Ca-fibrin. This factor is not present in the purified serum protein fractions and may have been destroyed or removed in their isolation.

13. The following equations illustrate the in vitro mechanism in the conversion of fibrinogen to fibrin:

Fibrinogen + Thrombase ⟿ T-fibrin

T-fibrin + Ionic Calcium + Serum factor ⟿ Ca-Fibrin

14. Heat coagulated fibrinogen, Ca-fibrin, T-fibrin and heat coagulated Ca-fibrin produce precipitins for fibrinogen. Heat coagulation of fibrinogen and the fibrins changes their original solubility in 0.03% hydrochloric acid and 0.5% sodium carbonate. From the results obtained with the immunologic methods, it would appear that the antigenic nucleus remains unaffected by clotting or by heat denaturation of fibrinogen, regardless of its change in solubility.

15. The fibrinogen and fibrin molecules do not contain either albumin or pseudoglobulin.

# VITA

KENNETH CARL ROBBINS

Born in Chicago, Illinois, September 1, 1917. Attended the Chicago Public Schools and graduated from Tuley High School, 1934. Received B.S. degree with honors from the University of Illinois, 1939; M. S. degree, University of Illinois, 1940.

Graduate student in Physiological Chemistry, University of Illinois College of Medicine, 1939-1944. Assistant in Physiological Chemistry, University of Illinois College of Medicine, 1941 to date.

Associate member of Sigma Xi, Chicago Colleges of the University of Illinois Chapter.